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Inactivated Eastern Equine Encephalomyelitis Vaccine Propagated in Rolling-Bottle Cultures of Chick Embryo Cells

FRANCIS E. COLE, JR.

U.S. Army Medical Research Institute of Infectious Diseases, Frederick, Maryland 21701

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A method was developed for the production of Eastern equine encephalomyelitis vaccine from virus grown in rolling-bottle cultures (840 cm² growth area) of chick embryo cells. The PE-6 strain of virus was propagated in chick embryo cell roller cultures maintained on serum-free medium 199 containing 0.25% human serum albumin and antibiotics (MM). A multiplicity of inoculum of 0.005 yielded acceptable titers of virus at a convenient harvest time of 18 to 24 hr and reduced the carry-over of extraneous material from the virus seed. Growth studies in which 100, 200, or 300 ml of MM was used showed that use of 300 ml of MM offered two advantages: (i) cytopathic effects were less at the 18- to 24-hr harvest time, thereby decreasing cellular material in the final product, and (ii) total virus yield was not substantially reduced, thus permitting large-scale production of virus for further processing. Studies on formalin inactivation at 37 C indicated that the virus was inactivated by 0.05% formalin within 12 to 16 hr and with 0.1% formalin within 6 to 8 hr. Antigen extinction tests in hamsters revealed excellent potency (e.g., median-effective-dose values of 0.069 to 0.012 ml) for both fluid and freeze-dried products. The advantages of the roller-bottle technique in vaccine production are discussed.

In a previous paper, a method was described for the production of a potent Eastern equine encephalitis (EEE) vaccine (3). The procedure yielded a safe, effective vaccine (1) that has been used successfully in more than 750 persons (P. J. Bartelloni, *personal communication*), but it was time-consuming and resulted in a low yield of vaccine. The technique required multiple handling of large numbers of chick embryo cell (CEC) cultures, and many man-hours of labor to produce a liter of vaccine. Moreover, repeated handling of the cultures increased the risk of contamination of the final product, particularly when larger lots of vaccine were desired.

Described in this paper is a method for the production of potent EEE vaccine by utilizing a single-passage harvest of virus propagated in rolling-bottle cultures of CEC.

MATERIALS AND METHODS

Virus. The PE-6 strain of EEE, obtained from the Walter Reed Army Institute of Research, has been described (3). A 20% suspension of infected chick embryos (torsos only) was prepared for use as production seed virus as well as challenge virus for potency assays. In the study of the multiplicity of inoculum (MOI), roller-bottle CEC cultures were inoculated with EEE seed virus at MOI of 5.0 to 0.0005 [i.e., 5.0 to 0.0005 mouse median lethal doses (LD_{50}) per cell] and maintained with 300 ml of maintenance medium. Samples of culture fluids were removed at regular intervals postinoculation and were assayed in mice. In all other studies, an MOI of 0.005 was employed.

Virus titrations. Virus suspensions were titrated using 3-week-old white mice, CD-1 strain (Charles River Mouse Farms, Wilmington, Mass.). Virus samples were diluted in cold phosphate-buffered saline, *p*H 7.2, containing 1% normal rabbit serum. Groups of six mice were inoculated intracerebrally (ic) with 0.03 ml of $\log_{10}$ dilutions of virus-containing fluids. Titration end points were determined by the method of Reed and Muench (5) and are expressed as LD_{50} per 1.0 ml.

Preparation of cell cultures. Nine-day-old chick embryos were minced and trypsinized by using conventional methods (6). The cells were suspended to a final concentration of 4×10^6 cells/ml in a growth medium consisting of Eagle's basal medium containing 10% calf serum and 1% glutamine plus 100 μg each of neomycin (USP) and streptomycin (USP) per ml. Rolling bottles (280 mm; Bellco Glass, Inc., Vineland, N.J.) with a growth area of 840 cm² were seeded with 170-ml volumes of the cell suspension, placed on a Bellco roller apparatus set at 0.4 rev/min,

and incubated at 35 C until confluent cell sheets were obtained (18 to 24 hr). The growth medium was then replaced with serum-free medium 199 containing 0.25% human serum albumin and antibiotics (MM). The CEC roller cultures were incubated an additional 20 to 24 hr at 35 C.

Infection of cell cultures. After the medium was poured off and the residual fluids were drained from the cell sheets, the infecting virus in 10 ml of MM was added and adsorbed for 1 hr at 37 C with the roller apparatus set at 0.7 rev/min. The infecting fluid was drained, and the specified volume of MM was added. Infected fluids were harvested at predetermined times without disturbing the cell monolayer.

Preparation of vaccine. Fluids from infected cultures were centrifuged at 900 × *g* for 30 min at 4 C and then passed through a membrane filter (0.45 μm pore size; Millipore Corp.) to remove cellular debris that might adversely affect inactivation of the virus. As previously indicated (3), no significant loss of infectivity occurs as a result of this clarification procedure. Within 1 hr of filtration, formalin (Formaldehyde, 37% assay) was added to the clarified viral harvests to final concentrations of 1:2000 (0.05%) or 1:1000 (0.1%). After addition of formalin, the fluids were thoroughly mixed by shaking, placed at 37 C, and agitated frequently. At the end of the desired period of inactivation, the material was held in closed vessels at 4 C for 15 days, during which time the vessels were shaken at least twice daily.

Assay of potency. Potency was determined by using a two-dose antigen extinction assay in hamsters as previously described (2). The potency is expressed as the median effective dose (ED_{50}), i.e., the volume of undiluted vaccine given in each dose of the two-dose series that protected 50% of the hamsters from death after challenge.

Tests for safety, toxicity, and sterility. Representative lots were tested for safety, toxicity, and sterility in accordance with procedures previously outlined (4).

Freeze-drying and final testing. Selected lots were subjected to final processing. Residual formalin, as determined by the NIH method (3), was neutralized with sodium bisulfite to a level of <0.01%. The materials were dispensed immediately into glass vials and freeze-dried in a cabinet dryer. The vials were sealed under vacuum with rubber stoppers and aluminum caps and stored at −20 C. Final products were tested for safety, sterility, toxicity, and potency (4).

RESULTS

Effect of MOI on optimal time of harvest. Optimal yields of virus were obtained at a convenient harvest time of 18 to 24 hr at an MOI of 0.005 (Table 1). This represented 10.0 ml of a 10^{-5} dilution of seed virus having a titer of $10^{10.8}$ mouse LD_{50}/ml. Although similar maximal titers were achieved at higher MOI levels, use of the 10^{-5} dilution decreased the amount of extraneous material carried over from the seed virus and permitted harvest as late as 24 hr postinoculation. Therefore, the 0.005 MOI was employed in all subsequent studies.

Effect of volume of maintenance medium on virus yield. Since previous experience in this laboratory suggested that EEE virus yield may be affected by the volume of medium employed, replicate cultures were infected at an MOI of 0.005 and maintained with 100 to 300 ml of MM. The results of titrations performed with fluids removed from these cultures at regular intervals are shown in Table 2. As indicated, between 12 and 30 hr postinoculation there were no substantial differences in the titers achieved using the three MM volumes. Of importance, however, was the observation that cultures maintained with 100 or 200 ml of MM showed far greater cytopathic effects than those maintained with 300 ml of MM.

Inactivation of virus. To determine the rate of inactivation, samples were taken at regular intervals during the inactivation period and titrated immediately in mice. Samples of viral harvests subjected only to 37 C for similar periods were included as controls. Shown in

TABLE 1. *Effect of multiplicity of inoculum (MOI) on propagation of Eastern equine encephalomyelitis virus in roller-bottle chick embryo cell cultures*

Hr post inoculation	Log10 median lethal dose per milliliter by MOI of				
	5.0	0.5	0.05	0.005	0.0005
6	≥7.0	≥6.7	≥7.0	>6.5	5.0
12	≥8.9	>9.0	≥9.0	>8.3	≥8.7
18	9.2	9.0	8.8	8.7	8.7
24	9.0	9.0	9.1	9.2	8.2
30	8.5	8.0	8.7	9.0	≤8.5

TABLE 2. *Effect of maintenance medium volume on propagation of Eastern equine encephalomyelitis virus in chicken embryo cell roller-bottle cultures*[a]

Time (hr) postinoculation	Log10 median lethal dose per milliliter by maintenance medium volume of		
	100 ml	200 ml	300 ml
6	7.2	7.0	6.8
12	9.9	9.1	9.1
18	10.0	9.5	9.1
24	10.3	9.8	9.3
30	9.3	9.3	9.3

[a] Multiplicity of inoculum, 0.005.

Table 3 are results of titrations performed on six of the vaccine lots produced.

With 0.05% formalin, complete inactivation generally occurred between 12 and 16 hr. With a final concentration of 0.1% formalin, inactivation was complete between 6 and 8 hr. As indicated, the rate of inactivation and therefore the exact killing time varied silightly among lots.

Effect of formalin concentration and length of inactivation period on vaccine potency. Both extended contact with formalin, particularly in higher concentrations, and exposure to temperatures above 0 C may result in antigenic damage in vaccines, thus lowering the potency of the final product. To evaluate the effect of these factors on EEE vaccines, 18 small lots of experimental vaccines were prepared by using either 0.05% or 0.1% formalin with inactivation periods of 24 to 48 hr. Listed in Table 4 are the results of potency assays performed on vaccines prepared during this study. As shown, acceptable vaccines were produced with either 0.05% formalin and a 36- or 48-hr inactivation period, or 0.1% formalin and shorter periods of inactivation. No correlation could be made between preinactivation titers, MM volumes, and vaccine potencies. Volumes of MM employed on cultures used to produce these vaccines ranged from 100 to 300 ml, whereas preinactivation titers of the viral harvests prior to formalin inactivation varied from $10^{9.3}$ to $10^{10.2}$ LD_{50}/ml.

TABLE 3. *Formalin inactivation[a] of Eastern equine encephalomyelitis virus at 37 C*

Time (hr) after formalin	Log10 median lethal dose per 0.03 ml by formalin concentration of		Virus[b] control
	0.05%	0.1%	
0	7.7–8.7[c]	7.7	7.7
2	2.9–4.4	1.6	
4	1.3–1.8	<1.0	
6	<1.0	<1.0	
8	<1.0	0[d]	4.0
10	<1.0	0	
12	<1.0	0	
16	0	0	
18	0	0	
24	0	0	
30	0		<2.0

[a] Determined by ic inoculation of adult mice with 0.03 ml.

[b] Blanks, not tested.

[c] Range observed at that time period on samples from different lots of vaccine.

[d] 0 indicates survival of 50/50 mice inoculated with undiluted vaccine.

TABLE 4. *Effect of formalin concentration and length of inactivation period on potency of Eastern equine encephalomyelitis vaccines*

Formalin concn (%)	Median effective dose (ml) by hr of inactivation			
	24	30	36	48
0.05	0.035[a] (0.028–0.045)	0.030[b] (0.012–0.069)	0.025[c] (0.020–0.034)	0.015
0.1	0.023	0.033	0.030	

[a] Mean of three lots; values in parentheses indicate range of values for median effective dose.

[b] Mean of seven lots.

[c] Mean of four lots.

Tests on selected lots which were subjected to final processing showed them to be safe, sterile, and nontoxic; no significant changes in ED_{50} values were noted in the freeze-dried products after storage for 18 months at −20 C.

DISCUSSION

In vaccine production it is desirable to reduce extraneous material in the final product to a minimum. This was accomplished in three ways: (i) use of a higher dilution of seed virus for inoculum, (ii) use of large volumes of maintenance medium, and (iii) clarification by centrifugation and filtration. The studies in which various MOI were employed indicate that the virus inoculum may be diluted to 10^{-5} (MOI = 0.005) without affecting the optimal time to harvest or the virus yield, while adding only minimal amounts of extraneous material from the virus seed. Further, when larger volumes of maintenance medium (e.g., 200 or 300 ml) were employed, satisfactory yields of virus were obtained not only at a convenient harvest time (18 to 24 hr), but also at a time during replication and release of virus when cell damage was minimal, thus reducing the cellular material carried over into the final product. Finally, clarification by centrifugation and filtration further aids in the removal of foreign materials.

Since no correlation could be made between preinactivation titers and vaccine potencies, even the material with the lowest titer ($10^{9.3}$ LD_{50}/ml) contained sufficient antigenic mass for the production of a potent vaccine. Because titers $\geqq 10^{9.0}$ LD_{50}/ml were consistently obtained in cultures maintained with 300 ml of medium, large volumes of high-titered viral harvests could easily be obtained by this method for large-scale production of vaccine.

The studies on formalin inactivation at 37 C clearly indicate that the exact time for complete

inactivation varied among lots, and must, therefore, be determined on an individual basis. This finding is of great significance in terms of standards outlined by the U.S. Public Health Service (4). Since these standards require formalin treatment at 37 C for a period of time three times that required to reduce virus to a nondetectable level, this would entail an inactivation period between 36 and 48 hr for the 0.05% vaccines described herein, and a period between 18 and 24 hr when 0.1% formalin is employed. No marked difference in potency was noted between vaccine lots prepared by using the two formalin concentrations and the four periods of inactivation described, some of which exceeded the minimum required period.

The method of vaccine production described in this paper possesses significant logistical advantages compared to the previously described procedure (3). The latter involved three successive, cumulative passages of virus-containing fluids in static CEC cultures. Since each roller bottle has approximately the same growth area as 11 to 12 plastic flasks and since cumulative passages do not appear to be necessary for the production of potent vaccines, much time and expense may be eliminated by use of the new procedure. The capability of the roller-bottle system to permit rapid production of large-scale lots of vaccine appears to be limited only by the capacity of the roller device employed.

Studies on freeze-drying, safety, sterility, and toxicity indicate that vaccine produced by the new method can be expected to be of the same quality and efficacy as the earlier vaccine (3).

Experiments now in progress indicate that the described procedure works equally well for the production of formalin-inactivated Western and Venezuelan equine encephalomyelitis vaccines.

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